# Superoxide dismutase mutations of familial amyotrophic lateral sclerosis and the oxidative inactivation of calcineurin

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**Abstract Approximately 10% of all familial cases of amyotrophic lateral sclerosis (fALS) are linked to mutations in the SOD1 gene, which encodes the copper/zinc superoxide dismutase (CuZnSOD). Recently, wild-type CuZnSOD was shown to protect calcineurin, a calcium/calmodulin-regulated phosphoprotein phosphatase, from inactivation by reactive oxygen species. We asked whether the protective effect of CuZnSOD on calcineurin is affected by mutations associated with fALS. For this, we monitored calcineurin activity in the presence of mutant and wild-type SOD. We found that the degree of protection against inactivation of calcineurin by different SOD mutants correlates with the severity of the phenotype associated with the different mutations, suggesting a potential role for calcineurin–SOD1 interaction in the etiology of fALS.** 

*Key words:* Calcineurin; Superoxide dismutase; Amyotrophic lateral sclerosis

## 1. Introduction

Mutations in the SOD1 gene, which encodes the enzyme copper/zinc superoxide dismutase, are associated with familial amyotrophic lateral sclerosis (fALS), a fatal neurodegenerative disorder affecting spinal cord and brain motor neurons [1]. It typically results in mortality within a few years after diagnosis. The mechanisms by which these mutations lead to the selective death of motor neurons are unknown. However, there is evidence for gain of function mechanisms such as enhanced peroxide or hydroxyl radical production [2], changes in the protein structure [3], and alterations in the metal-binding properties [4]. Today, more than 70 different mutations of the SOD1 gene are known ranging from mutations that are associated with a mild, slowly progressive form of fALS to mutations that cause a very rapid progression of the disease.

Recently, it was shown that SOD1 protects calcineurin, a serine/threonine-specific calcium/calmodulin-dependent phosphoprotein phosphatase, from inactivation by reactive oxygen species [5]. Calcineurin has been implicated in a wide range of diseases including ALS [6–9]. In addition, calcineurin is involved in a variety of cellular responses to calcium-mobilizing signals, including the control of ion channels, the release of neurotransmitters, and gene transcription [10].

In spite of the impressive amount of data collected during the last years, the primary molecular targets of the mutated SOD1 in fALS are still unknown. In order to address our hypothesis that calcineurin is one of the targets, we investigated the ability of four different copper/zinc superoxide dismutase (CuZnSOD) proteins, three mutant forms and the wild-type (wt) enzyme, to protect calcineurin from oxidative inactivation in vitro.

In this study, we report that the mutant SODs were significantly less protective against calcineurin inactivation, and that the severity of the loss of protection seems to be related to the severity of the respective form of fALS.

## 2. Materials and methods

### 2.1. Preparation of tissue extracts

100 ng–1 μg purified bovine brain calcineurin (Sigma Aldrich, Deisenhofen, Germany) or 100 μg homogenized tissue or cell extracts were used for standard calcineurin phosphatase assays. 100 μg cells or tissue were homogenized exactly as described [11]. Partially purified and redox-sensitive calcineurin was prepared by centrifugation of homogenates at 14 000 rpm at 4°C for 10 min (Eppendorf centrifuge 5417R) and separation of the resulting supernatant on a 1.5×10 cm Sephadex-G50 gel filtration column as described [11,12].

### 2.2. Recombinant expression and purification of wt and mutated CuZnSOD

CuZnSOD-pQE60 transformed *Escherichia coli* M15[pREP4] cells were plated on Luria–Bertani (LB)/ampicillin (100 μg/ml)/kanamycin (25 μg/ml) agar. Cultures were grown in 250 ml LB/ampicillin (100 μg/ml)/kanamycin (25 μg/ml) until $OD_{600}$ was 0.6. Constitutive leakage expression of human CuZnSOD was fully prevented by the repressor plasmid pREP4-lacI. Production of the human CuZnSOD fusion proteins was induced by addition of IPTG (1 mM). After 2 h, the bacterial cells were harvested by centrifugation (4000×*g*, 20 min), resuspended in 8 ml buffer A (20 mM Tris–HCl, pH 7.9, 5 mM imidazole, 500 mM NaCl) and homogenized by four freeze–thaw cycles and sonication on ice (Bandelin sonoplus GM70, 300 W, 3×10 s). The lysate was centrifuged (10 000×*g*, 20 min) and incubated with 750 μl Ni-NTA-agarose for batch affinity binding (1 h, 4 °C). The batch was applied to a 30 ml chromatography column, washed with 15 ml buffer A and subsequently with 8 ml buffer B (20 mM Tris–HCl, pH 7.9, 60 mM imidazole, 500 mM NaCl). C-terminal histidine-tagged CuZnSOD was eluted three times with 1.2 ml buffer C (10 mM Tris–HCl, 500 mM imidazole, 250 mM NaCl). Purity and identity of expression products was verified by immunoblotting or N-terminal protein sequencing after separation of 20 μl eluate in SDS–PAGE (discontinuous 12.5% SDS–PAGE).

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*2.3. Reactivation of CuZnSOD*

Wt and mutant SODs were purified and demetalated by Ni-NTA-affinity chromatography using imidazole (500 mM) as an eluant. In order to yield physiologically relevant active homodimeric CuZnSOD, the Ni-NTA eluate was subjected to ultrafiltration (3 kDa membrane from Centrex UF 0.5, Schleicher and Schuell, Dassel, Germany). For buffer changes, the samples were washed three times in refolding buffer (50 mM sodium citrate, pH 5.5, 1 mM dithiothreitol (DTT). After addition of $CuSO_4$ (200 μM) and $ZnCl_2$ (200 μM), the protein solutions were incubated at 8 °C or 37 °C for 7 days (250 μg/ml protein).

*2.4. SOD assay and activity staining*

Enzymatic activity of the CuZnSOD proteins was either analyzed by 10% native gel electrophoresis and activity staining with nitroblue tetrazolium dye, or by a quantitative spectrophotometrical assay according to published protocols [13,14]. Protein yields were determined by the Bradford method (Protein assay kit, Bio-Rad, Hercules, CA, USA). The concentration of purified CuZnSOD was determined spectrophotometrically using the extinction coefficient $\varepsilon_{265} = 1.84 \times 10^4$ $M^{-1}$ $cm^{-1}$.

*2.5. Atomic emission spectrometry*

For atomic emission spectrometry (AES), SOD proteins were washed five times in refolding buffer (Centrex UF 0.5, Schleicher and Schuell, Dassel, Germany). 100 μg protein in 1 ml were added to 0.5 ml $HNO_3$ and incubated at 95°C for 30 min. After cooling to room temperature, 10 ml $H_2O$ was added and the samples were analyzed with a ICP-VISTA RL CCD simultaneous ICP-AES.

Phosphoserine phosphatase assay was performed as described previously [5,15]. In brief, 40 μl recombinant or partially purified calcineurin was mixed with test buffer (40 mM Tris–HCl, pH 8, 0.1 M KCl, 0.4 mg/ml BSA, 0.67 mM DTT, 0.67 μM calmodulin, 1 μM FKBP and 0.5 μM okadaic acid for inhibition of phophotase A1 and A2). Calcium-induced redox inactivation of calcineurin was started by addition of 20 μl substrate buffer (0.67 mM $CaCl_2$, final concentration). At the indicated time points (2 min and 20 min), substrate was added (7.7 μM $^{32}$P-phosphorylated RII-peptide) and the reaction was incubated for 2 min at 30°C. Assays were performed in duplicate, repeated six times, and the inhibition of calcineurin activity by addition of 1 μM FK506 or cyclosporine was used to verify background activity for each reading point. The degree of protection by CuZnSOD against redox inactivation of calcineurin was determined by the addition of recombinant human wt, mutated CuZnSOD or of purified human erythrocyte CuZnSOD (200 ng or 800 ng, respectively). After 2 min at 30°C, the reaction was stopped with 100 mM potassium phosphate/5% TCA. The reaction mixture was passed through a 0.5 ml ion-exchange column (Dowex; AG 50W-X8, Bio-Rad) and the unbound phosphate eluted with 0.5 ml water. The quantity of released phosphate was determined by scintillation counting.

*2.6. Non-radioactive calcineurin assay*

5 μl of protein extract was added to a 96-well plate containing 25 μl of 2× assay buffer (Calbiochem), 5 μl okadaic acid (5 μm), 200 μg of SOD proteins and water ad 40 μl. Reaction was started by addition of 10 μl of phosphopeptide substrate. After 20 min incubation at room temperature, 50 μl of Green solution (Calbiochem) was added to each well and after additional 30 min, absorbance was read at 595 nm on a plate reader (Biolumin 960, Molecular Dynamics).

## 3. Results

To test the hypothesis that calcineurin is a target of mutated SOD1, we used recombinant wt enzyme and three variants associated with distinct clinical phenotypes. The mutations studied were associated with a rapidly progressive (amino acid substitution A4V), a classic (G93A) or a benign (D90A) course of ALS. In our expression, purification and reconstitution system, the D90A exhibited 78.5% of the specific activity, whereas the specific enzymatic activity of the G93A (22%) and A4V (13%) mutants CuZnSODs was profoundly reduced (Fig. 1). Activity staining of native electropherograms with NBT showed a corresponding pattern, with wt SOD showing the highest activity followed by the D90A mutant, whereas the activities of G93A and A4V mutants were strongly reduced (Fig. 1, lower panel). In line with the reduced zinc affinity of the mutants described by others [16,17], the metal content of the reactivated enzymes differed between the mutants (Fig. 1).

| | wild type | D90A | G93A | A4V |
|---|---|---|---|---|
| activity (u/mg) | 8,540+/-3,130 | 6,700+/-1,520 | 1,850+/-820 | 1,090+/-520 |
| Cu/Zn ratio | 0.81+/-0.01 | 2.23+/-0.04 | 2.51+/-0.04 | 1.51+/-0.01 |


Fig. 1. Characterization of the SOD1 proteins. The recombinant SOD1 proteins are characterized by a reduced activity and a reduced zinc content. Lower panel: Activity staining of recombinant SOD1 proteins. Activity was measured after 168 h of reactivation by a quantitative spectrophotometrical assay. Activity measurement, substrate gels and ion-content measurement were performed as described in Section 2. $n = 6$.

Incubation of calcineurin for 20 min at 30°C under aerobic conditions in the presence of calcium led to a rapid loss of phosphoserine phosphatase activity to 5–8% of baseline activity (Fig. 2a). This inactivation was $Ca^{2+}$-dependent and as effective as specific inhibition of calcineurin by FK506 (2.5% baseline activity). Oxidative inactivation could be prevented almost completely by addition of the $Ca^{2+}$ chelator EDTA, verifying the $Ca^{2+}$ dependency of the oxidative inactivation of calcineurin. Purified human erythrocyte CuZnSOD (1.7 U, Sigma-Biochemicals) or human recombinant wt CuZnSOD (200 ng, 1.7 U) preserved 58% of calcineurin baseline activity in the presence of $Ca^{2+}$ and oxygen (Fig. 2b), which is in keeping with recent findings by others [5]. Mutant hSODs (200 ng) were significantly less protective against calcineurin inactivation (Fig. 2b, light bars), and the degree of loss of protection was related to the severity of the respective form of fALS. The D90A mutation, found in a more benign form of fALS, which only gives rise to ALS in homozygous individuals, conveyed the most protection of calcineurin activity of the mutant SODs studied. The A4V mutant protein, which is associated with severely progressive fALS, was the least effective. The CuZnSOD bearing the G93A mutation, which is associated with standard/classic fALS, preserved only 22% of calcineurin activity, a loss of over 60% of the protection confered by wt SOD. This effect was not due to reduced SOD activity of the mutant SODs compared to wt: when the amounts of wt or mutant SOD were quadrupled (800 ng), the loss of protection of calcineurin activity was magnified, rather than reduced, for the fALS SOD mutants (Fig. 2b, dark bars), however, the differences in protection between 200 and 800 ng were not significant as tested by analysis of variance. While wt SOD maintained its protective effect on calcineurin activity and co-incubation of calcineurin with the SODs associated with benign (D9OA) and classic (G93A) fALS only showed a slight reduction compared to 200 ng of SOD, the SOD associated with the most severe form of fALS (A4V) resulted in a loss of 92% of calcineurin activity, meaning that over 80% of the protection of wt hSOD were lost due to this mutation. Repetition of this experiment with similar

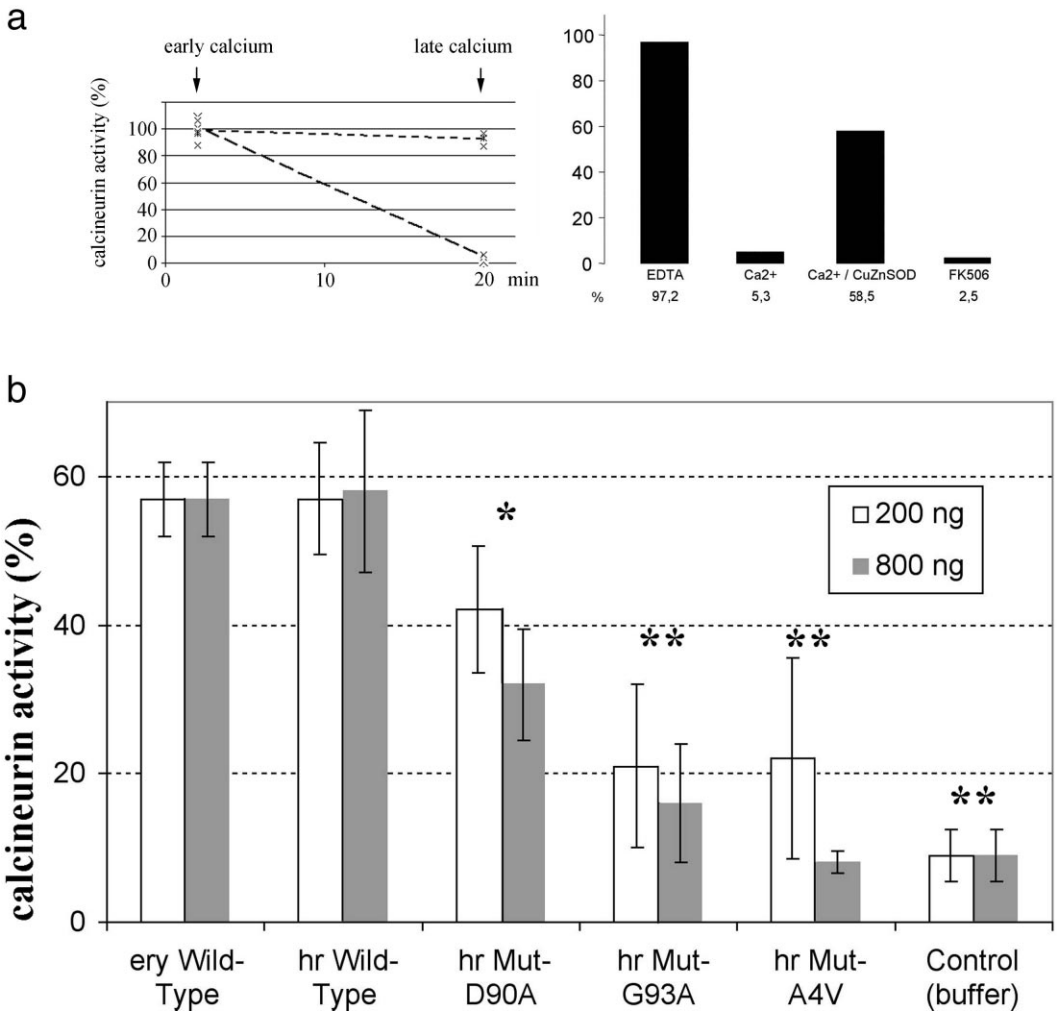


Fig. 2. Mutated CuZnSOD is less efficient in protecting calcineurin activity. a: Fast inactivation of calcineurin in the presence of calcium under aerobic conditions (left). CN activity was measured after 2 and after 20 min. Without early addition of calcium (upper dotted line), the CN activity remains stable until the start of the calcineurin measurement (second arrow), whereas after addition of calcium, the activity declines rapidly within 20 min. Addition of the chelator EDTA or co-incubation with CuZnSOD reduce the inactivation by oxygen (representative experiment, right), whereas addition of FK506, a calcineurin inhibitor, abolishes its activity. b: Calcineurin activity can be preserved by addition of erythrocyte (ery) SOD or recombinant human (hr) SOD. Recombinant mutant SODs associated with fALS are less protective. Addition of quadruple amounts of wt or mutant SODs (black bars) reduces the protective ability of the mutants even more. Calcineurin activity was assessed as described in Section 2. Values are given as mean ± standard deviation. $n = 6$; $^{*}P < 0.05$, significantly different from wt SOD by Student's $t$-test; $^{**}P < 0.01$, significantly different from wt SOD by Student's $t$-test.

amounts of units of each mutant (1.7 U) gave essentially the same results (data not shown).

To address the observation that increased amounts of mutant SOD compounded the loss of calcineurin activity, we tested a mixture of wt and G93A mutant (400 ng each). Calcineurin activity was measured by the dephosphorylation of the RII peptide as described in Section 2.

As shown in Fig. 3, a mixture of wt and G93A, as well as A4V mutations, showed an intermediate degree of protection. Our results thus demonstrate that mutant SODs can reduce the protection conferred by wt SOD. This observation supports the hypothesis that the SOD1 mutations found in fALS result in a negative gain of function, in this case in a reduction of the protection of calcineurin by wt SOD.

## 4. Discussion

How the different mutations in the ubiquitously expressed enzyme SOD cause the highly specific neuropathological phenotype associated with fALS remains elusive. Direct damage of cellular components by reactive oxygen species, but not superoxide, remains the predominant hypothesis. However, recent results argue against a simple accumulation of free radical or other damage in neuronal degenerations of various types, and rather suggest a 'one-hit' biochemical model in which the mutations impose a 'mutant steady state' on the affected neuron, and single events unrelated to the mutation randomly initiate the cell death [18]. The question of how this mutant steady state is induced remains unsolved, and the biochemical or genetic parameters are undefined.

Calcineurin is a calcium-dependent phosphoprotein phosphatase regulated by calmodulin. It has been implicated in a variety of cellular responses to calcium-mobilizing signals, for example the regulation of neuronal excitability by controlling the activity of ion channels, the release of neurotransmitters, synaptic plasticity, and gene transcription [10]. As calcineurin

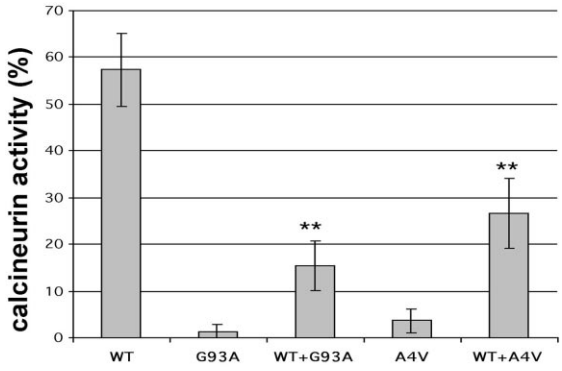


Fig. 3. A mixture of wt and mutant SODs confers intermediate protection. Addition of wt SOD (400 ng) to mutant SOD1s (400 ng) results in intermediate protective ability against oxidative inactivation. Calcineurin activity was assessed non-radioactively as described in Section 2. Values are given as mean ± standard deviation. $n = 4$; $^{**}P < 0.01$, significantly different from mutant SOD alone by Student's $t$-test.

comprises more than 1% of the total protein in the nervous system [19] and its activity can be protected by CuZnSOD [5], which itself accounts for 2% of brain protein, SOD and calcineurin are both among the most abundant proteins in neuronal cells. Therefore, calcineurin would be an ideal candidate for a proposed influence of superoxide dismutase mutations on signal transduction pathways leading to a mutant steady state finally resulting in neurodegeneration.

In this study, we report that mutated SODs loose their capacity to protect calcineurin from oxidative inactivation, as shown herein for three different mutations associated with different clinical phenotypes. In addition, the extent of loss of protection was related to the severity of the associated clinical phenotype. The SOD mutant showing the least protective effect (A4V) is associated with the most aggressive form of fALS, whereas an intermediate protective mutant (G93A) corresponded to an intermediate fALS progression rate and a mutation (D90A) linked to relatively benign form of fALS retained the highest protective ability of the three tested mutants. These results are in keeping with the observations of a reduced calcineurin activity in human neuroblastoma cells transfected with mutated SOD1 and in the forebrains of fALS-transgenic mice [9].

Moreover, when the amount of SOD protein was quadrupled in the assay, the wt maintained its protective ability, whereas the mutants showed an even more prominent loss of calcineurin protection. A mixture of both mutant and wt proteins showed an intermediate rate of protection. These results indicate that either the presence of the wt CuZnSOD protein, and not simply the presence of SOD activity, is needed for the protection of calcineurin from oxidative inactivation, or that the increased inactivation of calcineurin is mediated by mutated SOD-generated oxidative species different from superoxide, a hypothesis that has already been suggested by others [10,20]. Both possibilities would be in accordance with the notion that overexpression of wt SOD together with fALS-associated SOD is not protective [21], an observation that is strengthened by the fact that a mixture of wt and mutant SODs only confers an intermediate rate of protection. The partial protective effect of wt SOD when mixed with mutant SOD may be explained by the necessity of SOD to bind to calcineurin to exert its protective effect. Wt and mutant SODs therefore would compete for binding to calcineurin, so that part of the calcineurin molecules still could be protected by wt SOD.

It has to be noted, however, that the recombinant reconstituted SOD1 proteins used in this study show an abnormal zinc content, and therefore may not be completely refolded. A reduced affinity of SOD1 mutants for zinc is an observation that has already been made by others [16,17], and therefore a reduced zinc content of the recombinant proteins is not surprising. At the moment, we cannot evaluate to what extent this fact influences the observed results. However, our wt SOD1 gives exactly the same rate of protection as SOD1 purified from erythrocytes, and the D90A mutation, which shows a greatly reduced zinc content, shows a greater rate of protection than does the A4V mutant, whose zinc content is closer to that of the wt enzyme. This suggests that zinc content and protective ability are not directly related.

Independent of its relevance for ALS, our data give additional information that calcineurin is regulated not only by calcium, but also on the redox level. As other possible regulatory mechanisms such as cleavage of the autoinhibitory domain or inactivation by an endogenous FK506-analog have also been discussed, the picture of calcineurin regulation becomes increasingly complex.

Since the calcineurin inactivation is dependent on the presence of calcium, motoneurons, which are subject to glutamate-receptor-mediated $Ca^{2+}$ influxes, may be especially at risk for calcineurin inactivation. Moreover, calcineurin is responsible for the desensitization of NMDA receptor channels by dephosphorylating them. In light of our results, a reduction of calcineurin activity by mutant SODs would therefore lead to prolonged NMDA receptor-channel openings, and therefore to an increased $Ca^{2+}$ uptake, aggravating the situation even more. This would explain why glutamate potentiates the toxicity of mutant SOD1 in motor neurons [22]. As an inactivation of calcineurin has been shown early in cell death through apoptosis in some neuronal cell culture systems, and a higher Ca content in cells expressing mutant SODs or in mutant SOD1 transgenic mice has been observed [23–25], a dysregulation of the cellular calcium levels via calcineurin inhibition could explain the above-mentioned mutant steady state which renders motoneurons more susceptible to single random events that finally initiate cell death.

Our data show that the degree of calcineurin impairment depends on the type of mutation in CuZnSOD and corresponds to the severity of the disease. This suggests that altered calcineurin activity is at least partially involved in the disease progression in fALS. Protection of calcineurin against oxidative inactivation may therefore be a therapeutic approach in the treatment of ALS.

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